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INTERACTION BETWEEN PHOTOPERIOD, TEMPERATURE, AND CHILLING IN DORMANT LARVAE OF THE TREE-HOLE MOSQUITO, *TOXORHYNCHITES RUTILUS* COQ.

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Many temperate zone insects overwinter in a state of dormancy or diapause, where development ceases but other physiological and behavioral processes may remain active. For a great many insects, diapause may be terminated after prolonged chilling (Andrewartha, 1952; Lees, 1955; Williams, 1956) or by photoperiod (Lees, 1968; Danilevskii, 1965; Beck, 1968; Danilevskii, Goryshin, and Tyshchenko, 1970; Tauber and Tauber, 1976a). Even when photoperiod is important for the initiation and maintenance of dormancy, low temperatures may terminate diapause so that resumed development is independent of photoperiod (Danilevskii, *et al.*, 1970; Tauber and Tauber, 1976a). The two processes, response to day length and to chilling, are usually visualized as distinct, even though Bradshaw (1974) and Tauber and Tauber (1975) have shown that the critical photoperiod as well as the depth of dormancy may change during diapause. Neither of these studies clearly distinguished between the effects of low temperatures and those of prolonged exposure to short-day photoperiod.

The carnivorous tree-hole mosquito, *Toxorhynchites rutilus*, overwinters as a terminal (fourth) instar within the rot holes of deciduous trees in eastern North America (Holzapfel and Bradshaw, 1976). In Pennsylvania, the critical photoperiod and depth of diapause are considerably lower among larvae captured in January than among those caught in September or reared in the laboratory (Bradshaw and Holzapfel, 1975). These differences in the dormant state of individuals may be due to exposure to short days, to low temperatures, or to both. This study attempts to resolve to what extent the maintenance and depth of diapause is dependent upon photoperiod, temperature, and chilling.

Materials and Methods

All animals in the present study belong to the F_1, F_2, or F_3 generation of *Toxorhynchites rutilus septentrionalis* from Lahaska, Bucks County, Pennsylvania, 40°20′N. latitude, 75°04′W. longitude, and 180 m altitude. The P_1 generation was caught as diapausing larvae during September, 1973, and the subsequent colony maintained by induced mating (Trimble and Corbet, 1975; Holzapfel and Bradshaw, 1976). To initiate diapause, the larvae were reared under short-day conditions at $25 \pm 0.5°$ C (9L : 15D); they were maintained and experimentally used in individual 60×15 mm petri dishes. First and second instar larvae received freshly hatched brine shrimp (*Artemia salina*) as food; second and third instars received *Tubifex* worms. The larvae were fed, cleaned, and observed daily. To obtain a uniform cohort of individuals for the chilling experiment, only F_2 larvae which had hatched over an eleven day period and had spent 9–17 days as diapausing fourth instar larvae prior to the initiation of the experiments were used.

Illuminations included a single 15W tungsten lamp at a distance of 10–15 cm at 4 ± 2 C° and $7 \pm 1°$ C, two 40W cool white fluorescent lamps at a distance of 1.5 m at $16.5 \pm 1°$ C, and a single 4W cool white fluorescent lamp at a distance of 10–15 cm at 16.5 ± 1, 21 ± 1, and $25 \pm 1°$ C.

Tree-hole and air temperatures were recorded by a Rustrak Model 2133 thermistor-probe recorder, equipped with a time-sharing feature to record two temperatures at the same time.

Results

Development of unchilled animals at three constant temperatures

The effects of constant temperature on the development of unchilled animals were examined by comparing photoperiodic response and rates of development among larvae at 25, 21, and 16.5° C. Nine to fourteen larvae were exposed at each temperature and eight photoperiods, allowing 40 days for development at 25° C, 50 days at 21° C, and 80 days at 16.5° C. Figure 1A shows that the critical photoperiods were similar at all three temperatures, ranging from 12.9 hours at 25° C to 13.2 hours at 21° C with an intermediate value, 13.1 hours at 16.5° C. None of the larvae developed in response to 12 hours of light per day at any temperature; all of the larvae at each temperature developed within the experimental period when they were exposed to 14 or more hours of light per day. To obtain linear distributions from the sigmoid distributions shown in Figure 1A, percentage data were transformed first by setting values of 100 or 0 per cent equal to 99.99 or 0.01 per cent, respectively; then, % (transformed) $= \ln [(100 - \%)/\%]$. Regression of the transformed percentage of development on photoperiod and temperature indicated that development was significantly correlated with photoperiod ($F = 47.81$; $P < 0.001$) but not temperature ($F = 0.97$; $P > 0.3$), the former explaining 77% of the variation in the percentage of development, the latter less than 2%. Thus, temperature had little or no role in the termination of diapause, *per se*, among unchilled individuals of *T. rutilus*.

Figure 1B shows the rates of development at the two photoperiods (14L : 10D and 13L : 11D) for which data were available at all three temperatures. Two-

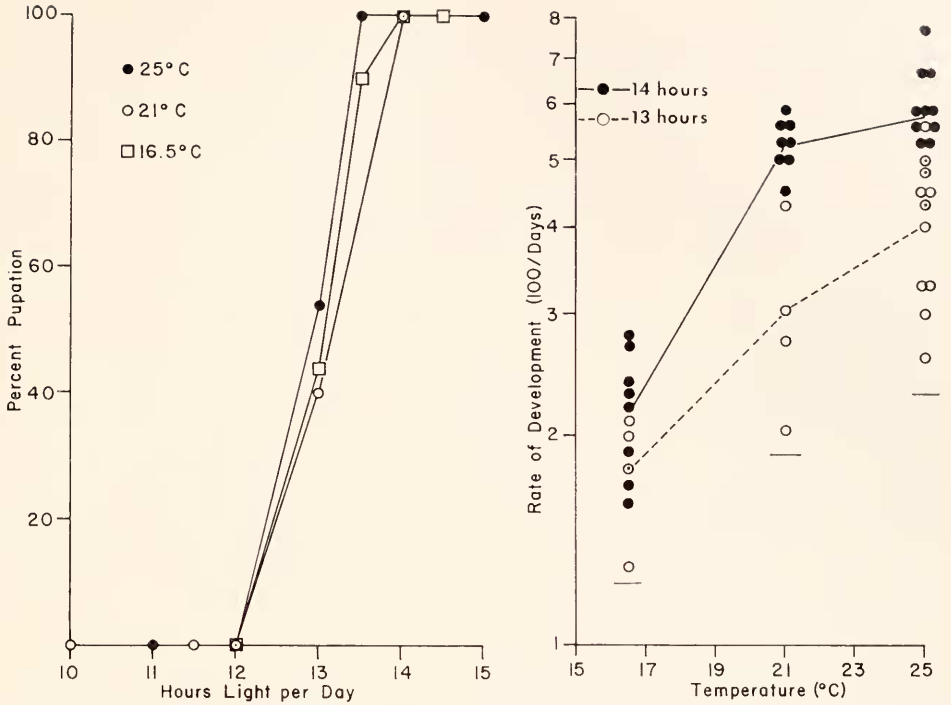

FIGURE 1. Effects of temperature and photoperiod on development. Left-hand graphs (A) show percentage of development in response to various photoperiods at 25°, 21°, and 16.5° C. Only photoperiod has a significant effect on percentage development. Right-hand plots (B) show rate of development in response to long (14 hour) and intermediate (13 hour) photophases at 25°, 21°, and 16.5° C. The horizontal line at each temperature shows the time interval of the experiment, *i.e.*, the minimum rate that could be observed at each temperature. Within this time interval, both temperature and photoperiod have a significant effect on rates of development.

way analysis of variance for unequal but proportional subclasses was carried out according to Sokal and Rholf (1969), after logarithmic transformation to achieve homogeneity of variance. Where subclasses were not proportional, individuals were eliminated by assigning each a number and excluding those numbers indicated by turning cards. Two-way analysis of variance revealed significant effects of both photoperiod ($F_{1, 31,} = 7.76$; $P < 0.01$) and temperature ($F_{2, 31,} = 16.23$; $P < 0.01$) and no significant interaction between temperature and photoperiod ($F_{2, 31} = 2.92$; $P > 0.05$). Thus, among unchilled larvae, there was a quantitative and additive effect of both photoperiod and temperature on the rate of development.

Chilling at four constant temperatures

The effects of prolonged exposure to low temperature were examined by placing 23 larvae each on a short-day regimen (9L: 15D) at $4 \pm 2°$, $7 \pm 1°$, $16.5 \pm 1°$, or $21 \pm 1°$ C for 159 days. During this time, only 3 out of 23 larvae survived at 4° C, while 19–21 survived at the higher temperatures (Table IA). The surviving

TABLE 1

Effects of chilling for 159 days at various temperatures followed by long (15L:9D) or short (9L:15D) days at 21° C.

	Chilling temperature			
	4° C	7° C	16.5° C	21° C
A. Per cent surviving 159 days chilling	13 (23)*	91 (23)	87 (23)	83 (23)
B. Per cent surviving 50 short days at 21° C after chilling	67 (3)	73 (11)	100 (11)	64 (11)
C. Per cent of survivors which develop within 50 short days at 21° C	0 (2)	38 (8)	0 (11)	0 (7)
D. Per cent larval survivorship on long days at 21° C after chilling	—	70 (10)	90 (10)	90 (9)
E. Rate of development on long days at 21° C after chilling	—	10.05 ± 2.12 (7)**	6.33 ± 1.18 (9)	5.26 ± 1.29 (8)

* Sample size is given in parentheses.
** Mean plus or minus two standard errors.

larvae were placed on long (15L:9D) or short (9L:15D) days at 21 ± 1° C. Of those exposed to the short-day regimen, 64–100% survived for at least 50 days, but only those which had been chilled at 7° C exhibited any development (Table IB–C). All of the larvae on the long-day regimen had either developed or died within 50 days. Larval survivorship ranged from 70–90% (Table ID), and mean rates of development (100/days to molt to a pupa) ranged from 5.26 to 10.50 (Table IE). Analysis of variance indicated significant differences between means ($F = 22.26$; $P < 0.01$). Duncan's multiple range test showed highly significant differences between the rate of development among larvae chilled at 7° C and those chilled at either 16.5° or 21° C. The difference in mean rates between larvae chilled at the latter two temperatures was not significant. These results indicated that prolonged exposure to 4° C produced substantial mortality and that the chilling "optimum" in terms of diapause termination or accelerated response to long-day photoperiod was above 4° C and below 16.5° C.

Effects of chilling on critical photoperiod

The effect of chilling on qualitative (critical photoperiod) as well as quantitative (rate) measures of development was assessed by chilling larvae at 7° C for up to six months and then by exposing them to various photoperiods at 21° C. A uniform cohort of 350 individuals of *T. rutilus* was reared at 21 ± 1° C, with nine hours of daily illumination. After 9–17 days as fourth instar larvae, 300 individuals were placed at 7 ± 1° C with a 9L:15D photoperiod, and ten larvae were exposed to each of the following photophases per 24 hour day: 10, 11.5, 12, 13, and 14 hours at 21 ± 1° C. At 45 day intervals thereafter, 50 larvae were removed from

the colder temperature and were subjected in samples of 10 each to various photophases at 21° C; photophases were determined from the results of ongoing experiments. After 180 days of chilling, samples of 14 larvae each were used to compensate for expected mortality.

Figure 2 shows that the critical photoperiod for development within 50 days of transfer to 21° C decreased consistently with prolonged chilling at 7° C. Prior to chilling, the critical photoperiod was 13.20 hours of light per day; after six months chilling, it had declined to 11.45 hours. In addition, after 180 days chilling, a substantial proportion of larvae developed in response to 9 or 10 hours of light per day.

The effects of chilling were distinguished from those of prolonged exposure to short days by comparing developmental response of unchilled individuals of *T. rutilus* with that of larvae chilled for six months after a total experimental time of 230 days (230 days at 21° C *vs.* 180 days at 7° C, plus 50 days at 21° C). After 230 days at 21° C (Fig. 2), the critical photoperiod for unchilled larvae was one hour longer than that of the animals chilled for six months and none of the larvae exposed to 10 hours of light per day had yet developed. These results showed that chilling, independent of short-day photoperiod, had a quantitative effect on the critical photoperiod and, after prolonged exposure, may have terminated diapause directly.

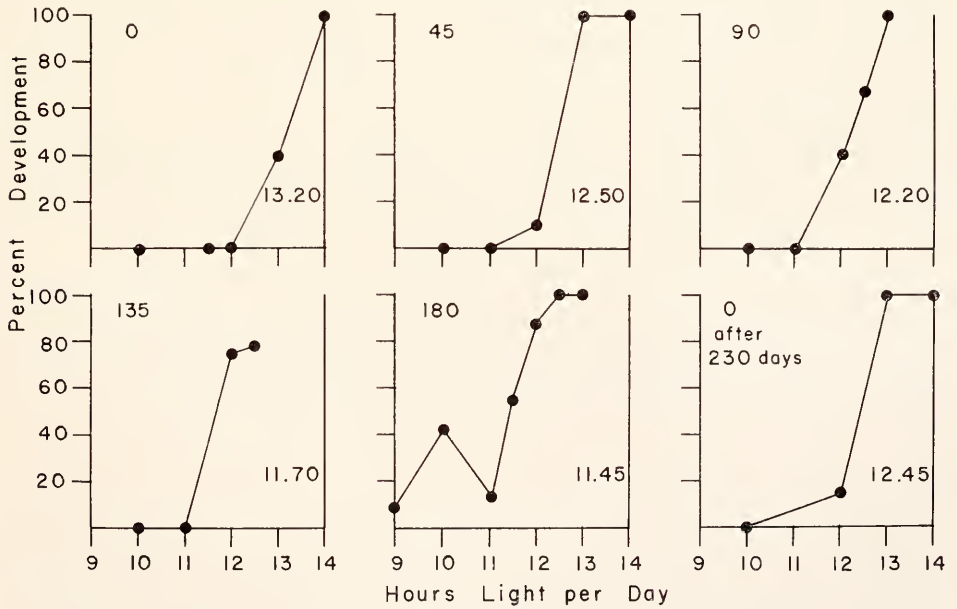

FIGURE 2. Qualitative effect of chilling on the termination of diapause. Larvae were chilled at 7° C for the number of days shown in the upper-left corner of each graph and then exposed to various photoperiods for 50 days at 21° C. Development was scored by noting the percentage of larvae which molted to pupae. Critical photoperiods are given in the lower right corner of each graph.

Effects of chilling on rates of development

The results in Figure 1A and Figure 2 showed that at photoperiods intermediate between long and short, an intermediate percentage of the sample population developed. These results dealt with development at the population level, and the question remained as to whether the population-statistical results were indicative of the response by individual larvae. Development through to pupation was an all-or-none response, but the rate at which each individual responded reflected the depth of its diapause and its physiological interpretation of environmental cues. Prior to chilling (Fig. 3A), 14 hours of light per day elicited a uniformly rapid rate of development and 13 hours, a more various and intermediate rate of development. With prolonged chilling, rates of development in response to the longest photoperiods progressively increased (Fig. 3F), as did the variability in response to intermediate daylengths (Fig. 3C–D). After 180 days chilling (Fig. 3E), rates of development separated into two distinct groups: those which molted to pupae within 15 days (at a rate of 6.7 or higher) and those which did not molt for 150 days (at a rate of 0.67). Among those larvae which molted within 150 days of transfer to 21° C (Fig. 3E), there was no significant difference in the rates at any photoperiod (analysis of variance: $F = 2.04$; $P > 0.05$). Prior to chilling, the mean rate of development in response to long days was 5.25 ± 0.25 (Fig. 3F) and after 159 additional short days at 21° C was still 5.26 ± 1.29 (Table IE). By marked contrast, there was a steady increase in the rate of development among the animals experiencing short days at 7° C and subsequently exposed to long days at 21° C (Fig. 3F). Finally, after 6 months at 7° C, some larvae developed in response to 9 and 10 hour photophases; yet, none of the larvae having experienced a 10 hour photophase continuously at 21° C developed for over 230 days (Fig. 2).

These results show that with progressive chilling, individuals lost their graded response to photoperiod and no longer interpreted any photoperiods as intermediate but only as long or short. At the same time, prolonged chilling decreased the depth of diapause and eventually effected its termination.

Chilling in *T. rutilus* plays a triple role. First, chilling can promote a response to progressively shorter daylengths, thus decreasing the critical photoperiod. Secondly, chilling can accelerate response to long days, thereby decreasing the depth of diapause. Thirdly, prolonged chilling can eventually terminate diapause directly, leaving subsequent morphogenesis independent of photoperiod.

Mortality after prolonged chilling

Mortality was low during chilling at 7° C (Table I). Mortality among unchilled larvae remained low for about 150 days, after which it increased sharply, reaching 36% by the end of the experiment (344 days) (Fig. 4). No appreciable difference in the pattern was observed among larvae chilled for 45, 90, or 135 days. Among larvae chilled for 180 days, there was an initial burst in mortality, which reached 30% within 30 days. By the end of the experiment, mortalities ranged from 36 to 40% regardless of chilling time.

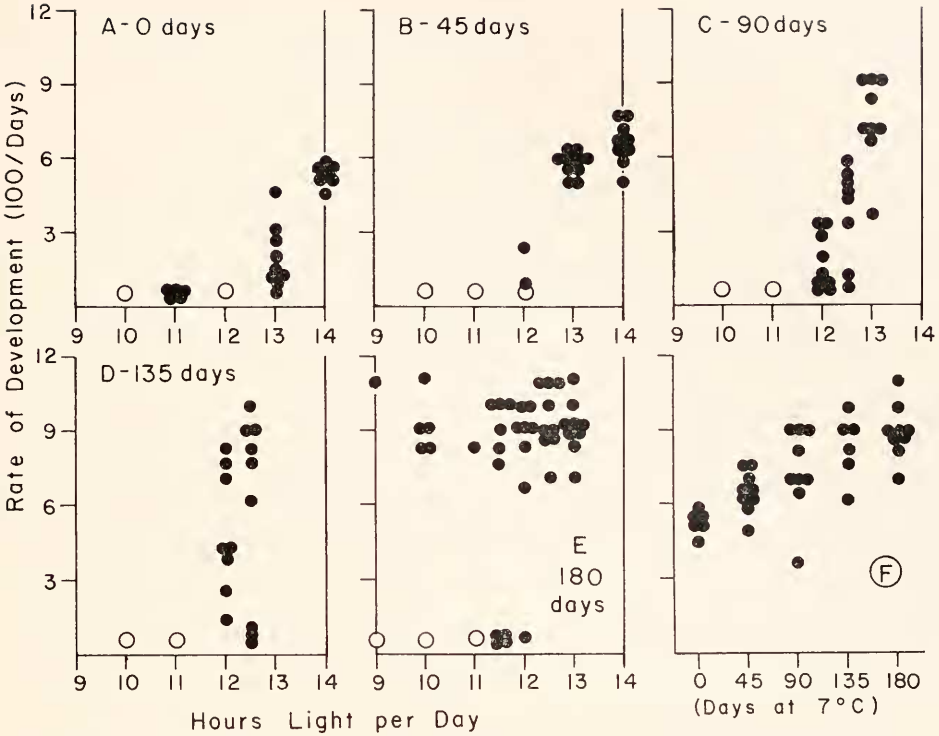

FIGURE 3. Quantitative effect of chilling and photoperiod on rates of development. Larvae and conditions are the same as in Figure 2. A–E show rates of development at various photoperiods after different chilling times; F, rates of development greater than 1.0 (100 days to molt to a pupa) at the longest photoperiod in A–E after different chilling times. Solid circles represent individuals; open circles represent six or more individuals.

Tree-hole temperature

To obtain tree-hole temperatures in nature and to correlate these temperatures with local ambient temperatures, a tree-hole was selected near Lahaska, Pa. Larvae of *T. rutilus* had been found in this hole for four consecutive winters, and large numbers of eggs had been observed there during the summer months. The hole lay about 4.2 m above the ground in an exposed maple tree and contained up to 5 liters of water. One probe of the recorder was placed in the water of the rot-hole, the other outside of the hole, about 1 cm from the bark on a shaded portion of the tree, 4.3 m from the ground. Temperatures were obtained continuously from May 14–20 and May 28 to June 15, 1976. During this period, air temperatures ranged from 3.0° to 30.0° C and tree-hole temperatures from 9.0° to 21.0° C. The temperature cycle in the tree-hole was of a lower amplitude and lagged that of the air. There was a close correlation ($R = 0.93$; $F = 69.7$; $P < 0.001$) between mean tree-hole temperature, TTH, and the weighted means of air temperature during the current, TA_t, and previous, TA_{t-1}, days: $TTH = 5.95 + 0.412TA_{t-1} + 0.188TA_t$. For practical purposes, the formula can be simplified to the form

$TTH = 6 + (\frac{1}{5})(TA_t + 2TA_{t-1})$, with no decrease in the coefficient of multiple correlation ($R = 0.93$) (Fig. 5A). Therefore, at least during the month of May and June, tree-hole temperature was closely correlated with air temperature.

Discussion

Prior to chilling, larval diapause in *T. rutilus* is maintained primarily by photoperiod (Figs. 1A and 2; Bradshaw and Holzapfel, 1975; McCrary, 1965). The critical photoperiod of this response varies as little as plus or minus 7.5 minutes over the range of 16.5° to 25° C (Fig. 1A). Moreover, the slopes of the lines between threshold and saturation do not appear to differ over these temperatures. The photoperiodic mechanism is thus highly temperature-compensated, maintaining a given set-point (critical photoperiod) and inherent accuracy (slope of the response curve) between 16.5° and 25° C. Although the photoperiodic clock imposes an all-or-none response on each individual over a wide range of temperatures, both temperature and photoperiod affect the rate of ensuing development (Fig. 1B). These differences in rates of development at various temperatures and photoperiods could be due to either variation in the rate of termination of diapause, *per se*, or in the completion of post-diapause morphogenesis. Regardless of the

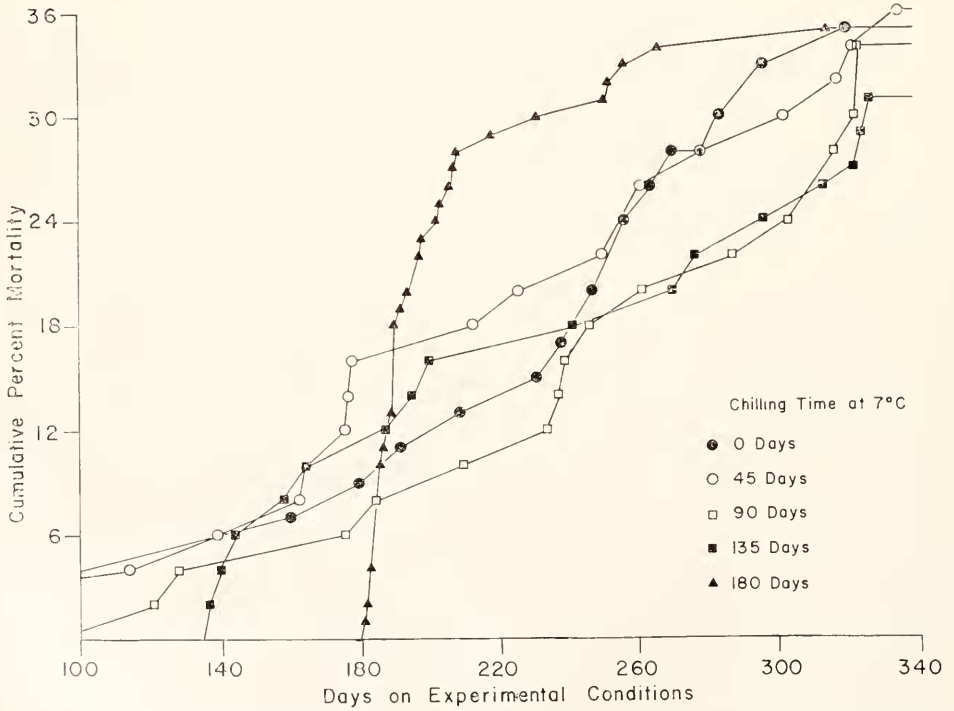

Figure 4. Mortality of larvae at 21° C (all photoperiods combined) after various chilling times. Days on experimental conditions refer to days since the start of the initial chilling and do not include rearing times up to the fourth instar.

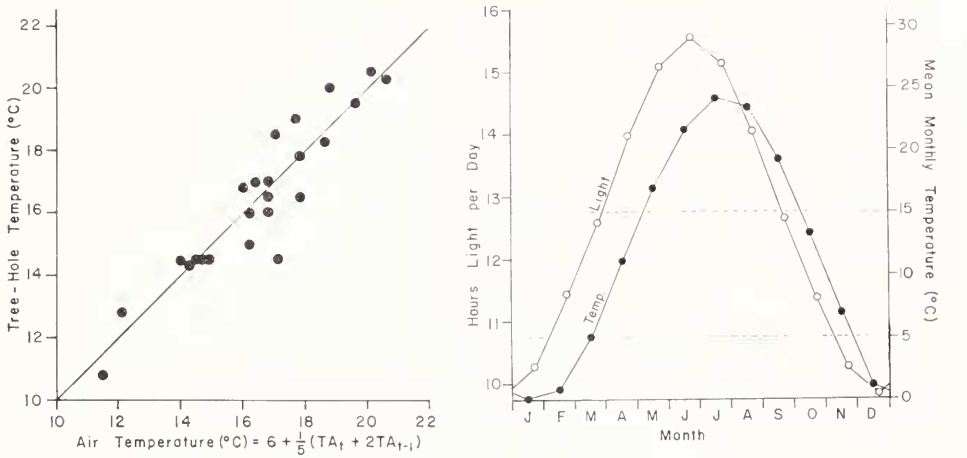

FIGURE 5. Light and temperature relationships near Lahaska, Pennsylvania. Left-hand plot (A) shows relationship between mean tree-hole temperature and air temperature. TA_t and TA_{t-1} are mean daily air temperatures of the current and previous day, respectively. Mean daily temperatures are calculated as the average of the daily maximum and minimum. Right-hand curves (B) show the seasonal march of both temperature (94-year average) and light (sunrise to sunset plus one civil twilight) for Philadelphia, Pennsylvania. Temeperatures were taken from the *Climatic Atlas of the United States* (United States Department of Commerce, 1968) and daylenghs from *Tables of Sunrise, Sunset, and Twilight* (Nautical Almanac Office, United States Naval Observatory, 1962).

mechanisms involved, an individual committed to resumed development by virtue of its photoperiodic clock will assume a more conservative rate at lower temperatures and photoperiods than at higher or longer ones.

In terms of rates of development (Fig. 3), chilling increases the responsiveness to all photoperiods. Thus, increased responsiveness to longer days results in a decrease in the depth of diapause; increased responsiveness to shorter and intermediate days results in a decrease in the critical photoperiod. Short of evoking the termination of diapause directly, these effects of chilling remain hidden until the larvae subsequently experience photoperiods at temperatures compatible with morphogenesis. It is tempting to draw a parallel between the accumulated facilitation of development in *T. rutilus* and the summation of covert effects of ecdysone in the fleshfly, *Sarcophaga peregrina* (Ohtaki, Milkman, and Williams, 1968). In *S. peregrina,* ecdysone is rapidly inactivated, but its effects accumulate so that even though the titer of ecdysone at any one time remains low, the accumulated effects render the target tissues sensitive to progressively less additional hormone. Analogously, in *T. rutilus,* chilling temperatures may cease to prevail, but the summation of their covert effects remains. Relative to unchilled larvae, fewer days additional chilling or shorter daylengths are then required to terminate diapause. Since both chilling and photoperiod may affect the brain directly in other insects (Williams, 1956; Williams and Adkisson, 1964), the covert effects of these same factors may summate in the brain of *T. rutilus,* rendering it more responsive to subsequent stimulation or, eventually, terminating diapause.

The termination of diapause after prolonged chilling may serve as a safety valve, since this role is expressed only when the larvae are approaching the limits of tolerance to long exposure at low temperature (Fig. 4). The adaptive significance of the interaction between temperature and photoperiod is not so apparent. As shown in Table I, chilling has its greatest effect above 4° and below 16.5° C. Even though there is a significant chilling effect at 7° C and a substantial reduction of the critical photoperiod produced by the same temperature (Figs. 2 and 3), the optimum may be somewhat higher. Figure 5B shows mean monthly temperature for Philadelphia, Pennsylvania, approximately 60 km from Lahaska. Figure 5A shows that the tree-hole temperature is closely correlated with air temperature during May and June. Although tree-hole and air temperature data for all seasons were not obtained, direct observation revealed that the tree-hole from which the recordings were made froze over for most or all of January, indicating that temperatures of 4° C or lower prevail at this time of the year. If 5–15° C may be taken as the range of temperatures which are likely to be important for promoting the chilling reaction, it is apparent that chilling is an autumnal and vernal, rather than an hibernal process. The adaptive significance of chilling as an environmental cue must then be interpreted in view of fall and spring conditions.

The interaction between the chilling and photoperiodic mechanisms in *T. rutilus* may represent an adaptive compromise between selection, due to long-term climate on the one hand and immediate weather conditions on the other. In temperate latitudes during the fall, the consequences of misinterpreting environmental cues are necessarily drastic. Reliance upon cues which maximize average fitness over a number of years will result in the correct phenotype, *i.e.*, diapause (Levins, 1969; Cohen, 1970). Photoperiod is a precise geophysical cue and is used by many insects in late summer and fall for the initiation and maintenance of diapause under otherwise favorable conditions of food and temperature (Lees, 1968; de Wilde, 1962; Danilevskii, 1965; Danilevskii, *et al.*, 1970; Beck, 1968; Tauber and Tauber, 1976a). In the spring, daylength is equally precise but is a less reliable indicator that spring is actually progressing. Spring temperatures, however, are themselves a reflection of individual vernal weather. Cold spring temperatures, below 5° C, would delay the influence of chilling on the photoperiodic mechanism; the larvae would remain deep in diapause, and the critical photoperiod would remain high. The larvae would then be more conservative in their response to subsequent photoperiods, and longer days would be required to elicit a given rate of development than if milder conditions, above 5° but still below 15° C, had prevailed. Consequently, a late winter or cold spring would result in delayed development. An interactive chilling-photoperiod mechanism thus combines the temperature-compensated precision of photoperiodism with a complimentary mechanism that tracks weather during individual springs. Variations on this theme may result in either a combination of events necessary to terminate diapause for all individuals in the population (homeostasis) (Stross, 1971; Adkisson and Roach, 1971; Wellso and Adkisson, 1966; Norris, 1965; Ryan, 1975; Kamm, 1972; Tauber and Tauber, 1976b) or a variety of different events necessary to terminate diapause in different individuals (polymorphism) (Bradshaw, 1973; Waldbauer and Sternberg, 1973; Morris and Fulton, 1970).

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Summary

1. Unchilled, diapausing larvae of *Toxorhynchites rutilus* rely on photoperiod for the maintenance of diapause. The photoperiodic clock is temperature-compensated between 16.5° and 25° C, maintaining both a similar set-joint and inherent accuracy over this range. The rates of development among larvae terminating diapause are dependent upon both temperature and photoperiod.

2. Chilling of dormant *Toxorhynchites rutilus* can promote response to progressively shorter daylengths, thus decreasing the critical photoperiod. Chilling can also accelerate response to long days, thereby decreasing the depth of diapause and, after prolonged exposure, can eventually terminate diapause directly, leaving subsequent morphogenesis independent of photoperiod.

3. The optimal temperature for these effects of chilling is above 4° C, below 16.5° C, and may lie around 7° C.

4. Temperatures between 5° and 15° C are vernal and autumnal rather than hibernal. The interaction between chilling and photoperiod may then represent an adaptive compromise between selection due to long-term climatic trends and the vagaries of spring weather.

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